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Fully Automated LC-MS/MS Analysis of Anticoagulants Using a Stable Isotope Labelled Internal Standards

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Overview

- This work describes a fully automated method to quantify 9 anticoagulant drugs in plasma.
- The method has been designed to meet the needs of monitoring anticoagulant drugs in routine clinical pathology.

Introduction

Novel oral anticoagulants (NOACs) are, as an alternative therapy to vitamin K antagonists, used frequently to treat and prevent thromboembolism. Their precise quantitation is necessary to identify the presence/absence of an anticoagulant effect or to determine the concentration of drug that may be helpful for patient management. Furthermore, anticoagulants screening is required for interventional emergency, emergency bleeding and programmed surgery for the elderly.

Such analysis is mainly done by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To streamline the workflow, we developed a complete solution including stable isotope labeled standards for better precision and accuracy, and investigated the use of a fully automated sample preparation system coupled online with LC-MS/MS.

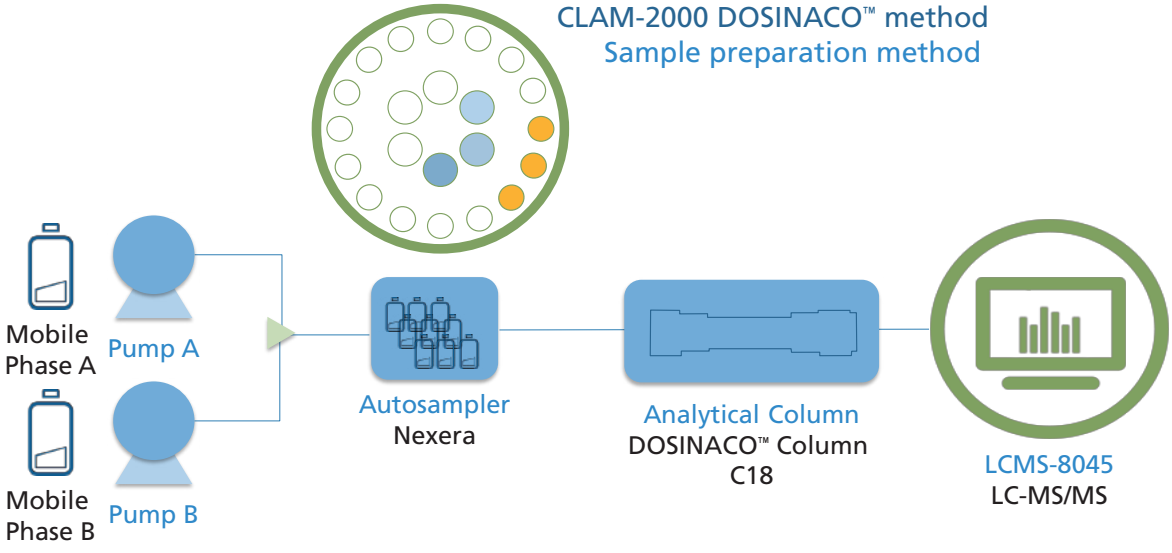

Figure 1. Schematic representation of the CLAM-2000 LC-MS/MS method for anticoagulant drugs in plasma

Methods and Materials

To demonstrate that this multi-analyte approach, with a fully automated system LC-MS/MS, can be used as a walk-away unit, we have used a novel kit for anticoagulants analysis called DOSINACO™ (Alsachim, France). The kit includes 9 analytes (Acenocoumarol, Apixaban, Argatroban, Betrixaban, Dabigatran, Edoxaban, Fluindione, Rivaroxaban and Warfarin) and their corresponding in-house stable isotope labeled standards. Fully automated sample preparation system CLAM-2000 (Shimadzu, Japan) was programmed to perform protein precipitation followed by filtration and sample collection. The sample is transported from CLAM-2000 to HPLC without human intervention. Separation was achieved within just 7 minutes using a C18 column maintained 50°C on a UHPLC system (Nexera-X2, Shimadzu, Japan). Data acquisition was performed on triple quadrupole mass spectrometer LCMS-8045 (Shimadzu, Japan). Calibration curves were prepared by internal standard method.

HPLC Conditions	
Analytical column	: DOSINACO™ Column C18 2,1x50 mm, 5 µm
Mobile Phase A	: DOSINACO™ Mobile Phase A
Mobile Phase B	: DOSINACO™ Mobile Phase B
Rinse solution	: (R0) DOSINACO™ System Cleaning Phase
(Internal & External)	(R1) DOSINACO™ Mobile Phase B
Flow rate	: 0.5 mL/min
Oven temperature	: 50 °C
Injection volume	: 2 µL

MS Conditions	
Ionization	: ESI Positive
Interface voltage	: 2.5 kV
DL temp.	: 200 °C
Heat Block temp.	: 400 °C
Interface temp.	: 400 °C
Nebulizer gas flow	: 3 L/min
Drying gas flow	: 5 L/min
Heating gas flow	: 15 L/min

Time program

Time (min)	event	(%)
0.00	Pump B conc.	2
0.50	Pump B conc.	2
2.50	Pump B conc.	50
3.00	Pump B conc.	98
5.00	Pump B conc.	98
5.01	Pump B conc.	2
7.00	Stop	

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MRM transition

Moleculs	Transitions MRM (1)	Transitions MRM (2)
Acenocoumarol	354.10>163.10	354.10>296.10
Apixaban	460.20>443.20	460.20>199.10
Argatroban	509.20>384.20	509.20>70.00
Betrixaban	452.10>324.10	452.10>279.10
Dabigatran	472.20>289.20	472.20>144.20
Edoxaban	548.20>366.20	548.20>152.10
Fluindione	241.10>175.10	241.10>194.10
Rivaroxaban	436.10>145.10	436.10>231.10
Warfarin	309.10>251.10	309.10>163.10

Moleculs	Transitions MRM (1)	Transitions MRM (2)
[$^{2}H_4$]-Acenocoumarol	358.10>167.10	358.10>300.10
[^{13}C, $^{2}H_8$]-Apixaban	469.20>452.20	469.20>199.10
[$^{13}C_6$]-Argatroban	515.20>390.20	515.20>70.00
[$^{13}C_6$]-Betrixaban	458.10>330.10	458.10>285.10
[$^{13}C_6$]-Dabigatran	478.20>295.20	478.20>144.20
[$^{2}H_6$]-Edoxaban	554.20>372.20	554.20>158.10
[$^{13}C_6$]-Fluindione	247.10>181.10	247.10>200.10
[$^{13}C_6$]-Rivaroxaban	442.10>145.10	442.10>237.10
[$^{2}H_6$]-Warfarin	315.10>257.10	315.10>163.10

Results

LC-MS/MS has then become the gold standard due to its specificity, precision and sensitivity. However, its use in the clinical laboratory has been restricted for several reasons, including high instrument costs, the need for development of instrument-specific analytical protocols and the need for skilled technicians.
The fully automatic LCMS preparation unit was programmed to perform sample extraction and protein precipitation followed by filtration and sample collection. The filtrated sample was then automatically transported using an arm to the HPLC for LC-MS/MS analysis and no human intervention was required. Fully automated sample preparation method was finally compared with manual sample preparation method by analyzing several samples spiked with 9 anticoagulants at various concentration levels.

Samples preparation for manual handling

1. Put 50 µL of samples/calibrators in 1.5 mL microtube
2. Add 25 µL of Internal Standard
3. Add 350 µL of Extraction buffer
4. Shake for 1 min
5. Centrifuge at 15,000 g for 7 min
6. Transfer 200 µL of supernatant to vial

Samples preparation for CLAM-2000

1. Take 20 µL of Extraction buffer to sample cup
2. Add 20 µL of samples/calibrators
3. Add 155 µL of Extraction buffer
4. Add 12.5 µL of Internal Standard
5. Shake for 2 min at 1,900 rpm
6. Filtrate for 2 min

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A panel analysis of 9 anticoagulants using an automated sample preparation system, seamlessly integrated on-line with LC-MS/MS, and combined with DOSINACO™, demonstrates the capability to use a standardized platform for therapeutic drug monitoring even for non-expert users of Mass Spectrometry. We carried out concurrent analysis over a range of concentrations in 10 µg/L to 500 µg/L for NOACs and 100 µg/L to 5,000 µg/L for traditional anticoagulants. The calibration curves that were generated had linear regression values of r^2 >0.99 for each curve. The classical LC-MS method limitations are thus dramatically decreased, and it is eliminating potential errors traditionally associated with manual sample handling. Furthermore in order to estimate the precision of the method, reference plasma control DOSINACOTM were analyzed several times (3 replicates per day during 3 days). For all anticoagulants, the CV and deviation values were within acceptable analytical ranges.
Sample preparation and LC/MS/MS analysis can be performed in parallel to accelerate throughput using CLAM-2000.

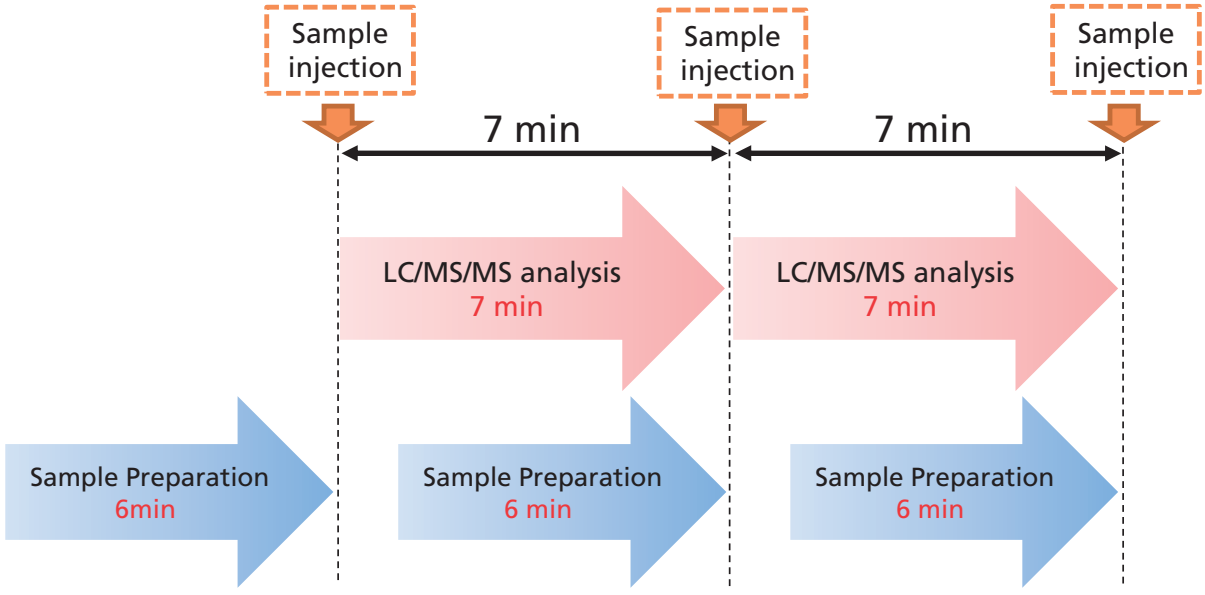

Figure 2. Analytical flow with parallel processing

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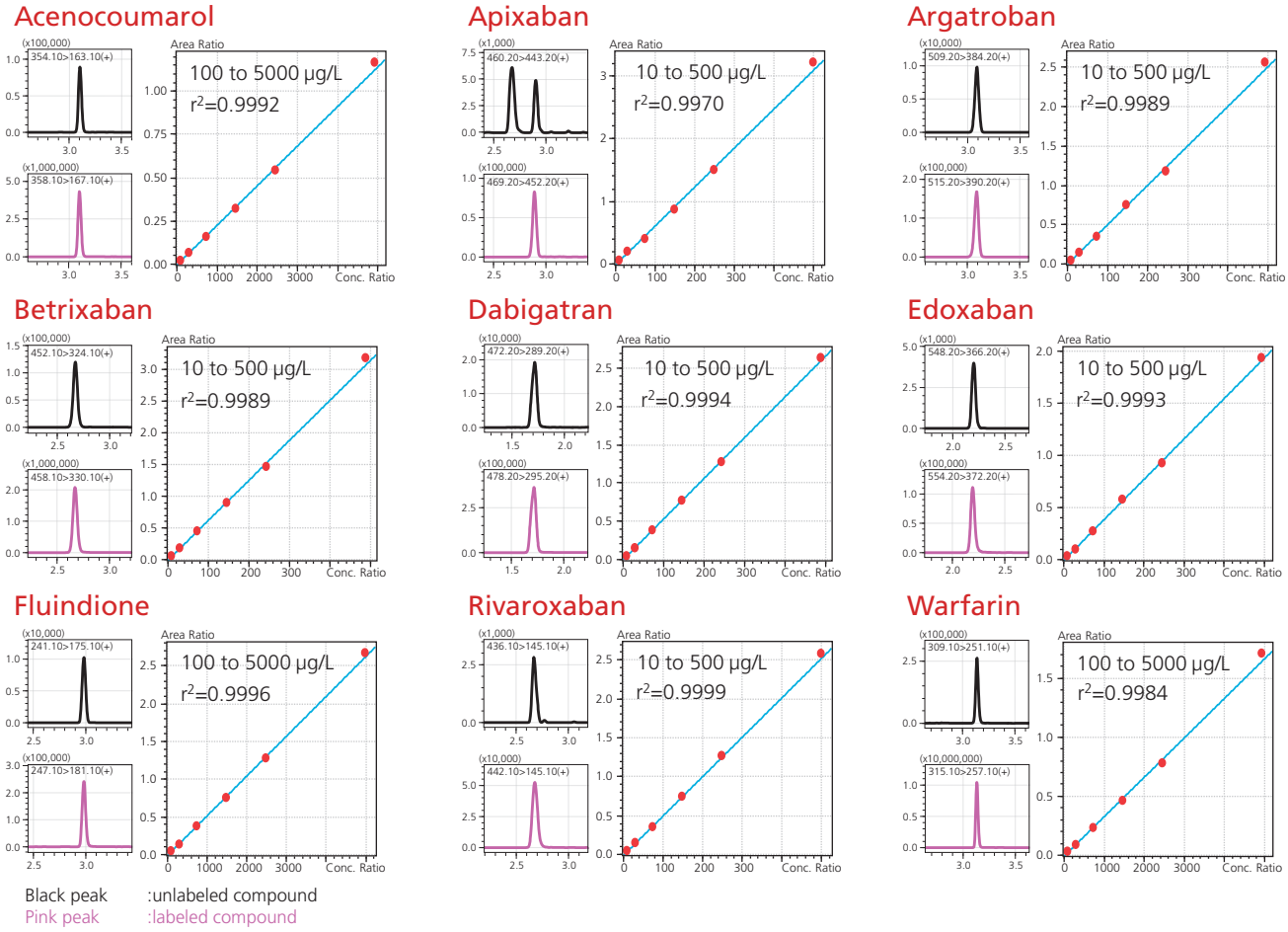

Black peak :unlabeled compound
Pink peak :labeled compound

	Acenocoumarol				Apixaban				Argatroban			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	185.9	858.3	1765.9	3536.8	17.8	79.0	162.3	323.3	17.2	81.8	172.6	333.6
CV (%)	1.8%	1.9%	1.7%	2.2%	10.6%	5.4%	6.0%	5.8%	5.5%	3.1%	3.2%	3.6%
Deviation (%)	0.6%	1.7%	2.9%	-0.4%	-1.0%	-4.2%	-4.7%	-4.3%	-2.9%	1.4%	0.9%	-4.6%

	Betrixaban				Dabigatran				Edoxaban			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	18.4	81.6	167.3	338.2	17.8	82.6	168.7	336.4	17.5	84.6	174.3	361.3
CV (%)	3.8%	1.6%	1.6%	1.9%	2.4%	1.3%	1.2%	1.0%	8.7%	5.3%	4.7%	5.2%
Deviation (%)	3.3%	1.4%	-1.5%	-0.9%	-0.1%	1.6%	-0.1%	-2.0%	-6.2%	0.1%	0.0%	1.7%

	Fluindione				Rivaroxaban				Warfarin			
Level	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
Average (µg/L)	198.1	856.6	1795.8	3691.4	18.3	81.2	170.8	345.2	177.8	819.5	1692.1	3453.5
CV (%)	6.4%	2.9%	2.9%	4.2%	7.4%	5.6%	6.0%	8.1%	1.8%	1.4%	1.0%	2.2%
Deviation (%)	9.1%	0.1%	1.8%	-1.1%	-2.7%	10.3%	-0.5%	5.2%	-0.1%	2.2%	0.6%	-0.4%

N=9 (3 replicates per day during 3 days)

Figure 3. MRM chromatograms (at calibrator level 1), calibration curves and summary of 9 anticoagulants

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Conclusions

- Fully Automated sample preparation procedure led to suitable results for the quantitation of anticoagulants thus eliminating all manual preparation steps.
- The novel system workflow results in easier and safer operation for users even without Chromatography and Mass Spectrometry experience, thus reducing risk of exposure. It allows to access and analyse hundreds of analytes on the same system without any modification thus improving the quality of service delivered to doctors for quick decision.
- The system would be suitable for emergency analysis as it is simple to use and can give quickly a presence of anticoagulants.

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